REGULATION OF EPIDERMAL GROWTH FACTOR RECEPTOR UBIQUITINATION AND TRAFFICKING BY THE USP8/STAM COMPLEX

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SUPPLEMENTARY FIGURE LEGENDS

FIG S1. USP8 regulates ligand-mediated EGFR degradation through an Hrs-dependent pathway.
(A) USP8 knockdown results in an accelerated EGFR turnover in response to EGF. HeLa cells transfected with pSilencer vector generating either control siRNA (*pS-Control*) or siRNA directed against human USP8 (*pS-USP8*) were serum-starved and treated with 2.5 ng/ml EGF for the specified length of time. Western blot analyses of total cellular EGFR, USP8 and transferrin receptor (*TrfR*) observed throughout the treatment time-course are shown. **(B)** USP8 modulates EGFR degradation through an Hrs-dependent pathway. Cells transfected with *pS-Control* or *pS-USP8* in combination with either control oligonucleotides (*siControl*) or oligonucleotides directed against human Hrs (*siHrs*) were serum-starved and treated in the absence (-) or presence (+) of 2.5 ng/ml EGF for 1 hr. Western blot analyses of relevant endogenous proteins from a representative experiment are shown.

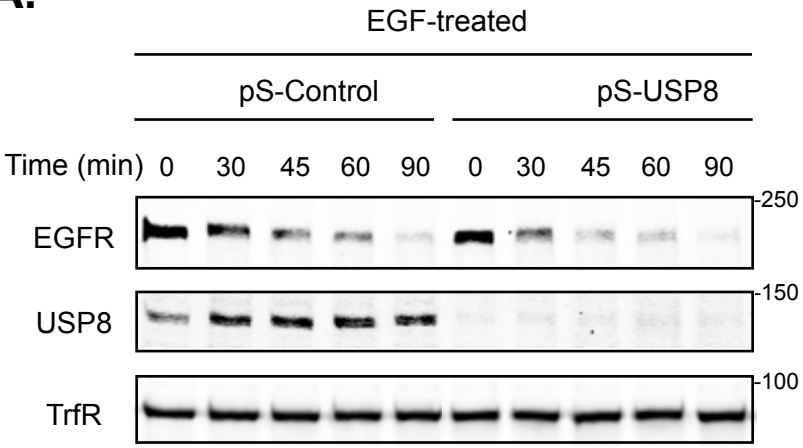
FIG S2. EGFR traffics through the STAM-positive endosome and the SH3 domains of STAM adaptor proteins modulate ligand-induced ubiquitination of EGFR.
(A) EGFR localizes to STAM1-positive endosomes quickly following activation with ligand irrespective of USP8 activity. Cells transfected with either wild type *USP8-CFP* or catalytically inactive *ΔC-CFP* were serum starved and treated in the presence of EGF for 10 min. Following treatment, cells were fixed and immunostained against endogenous STAM1 (*green*) and EGFR (*red*) proteins. Representative CFP-positive cells are shown with the scale bar corresponding to 10 microns (CFP fluorescence not shown). **(B)** Overexpression of STAM proteins leads to increased ubiquitination of EGFR in an SH3 domain-dependent manner. Cells co-transfected with HA-ubiquitin (*HA-Ub*) and vector, wild type *STAM1* and *STAM2* or SH3 domain mutants, *STAM1-WA* and *STAM2-EEAA*, were serum-starved and treated in the presence of 10 ng/ml EGF for 10 min. EGFR was immunoprecipitated and ubiquitination was analyzed by western blot against HA. **(C)** Quantification of data in (A); n=3, error bars correspond to SD.

FIG S3. Identification of three RXXK motifs within the central region of USP8.
(A) A far-western SPOTs peptide array scan of mouse USP8 protein sequence probed with GST-Gads-SH3C visualized on film by chemilluminescence using an anti-GST HRP-conjugated antibody. All peptides were 12 amino acids in length with a window of 5 amino acids scanning the entire USP8 protein sequence. **(B)** Glutathione precipitation of wild type *Flag-Gads* or its C-terminal SH3 domain mutant, W300A (*Flag-Gads-WA*), with GST-fusion proteins containing the RXXK motifs of Slp76 or USP8. **(C)** Table of equilibrium dissociation constants (K_D) calculated on the basis of 3 independent FP experiments.

FIG S4. USP8 modulates ubiquitination of STAM but not Grb2.
HeLa cells co-transfected with HA-ubiquitin (*HA-Ub*) and vector, wild type (*WT*) or catalytically inactive USP8 containing either intact (*ΔC*) or mutated (*ΔC-R3K*) RXXK motifs were grown in complete media. Endogenous **(A)** STAM1 or **(B)** GRB2 proteins were immuno-precipitated as indicated and their ubiquitination status was assessed by western blot against HA. Comparison of the effects of *ΔC* and *ΔC-R3K* on STAM1 ubiquitination are shown in Fig. 5E.

FIG. S1

A.


EGF-treated
pS-Control
pS-USP8
Time (min) 0 30 45 60 90 0 30 45 60 90
EGFR
USP8
TrfR
250
150
100

B.

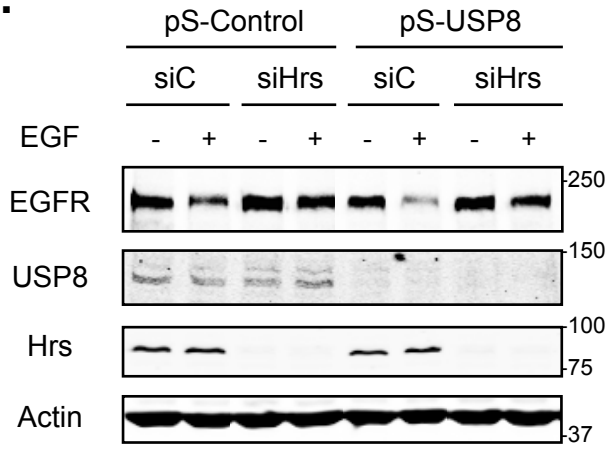
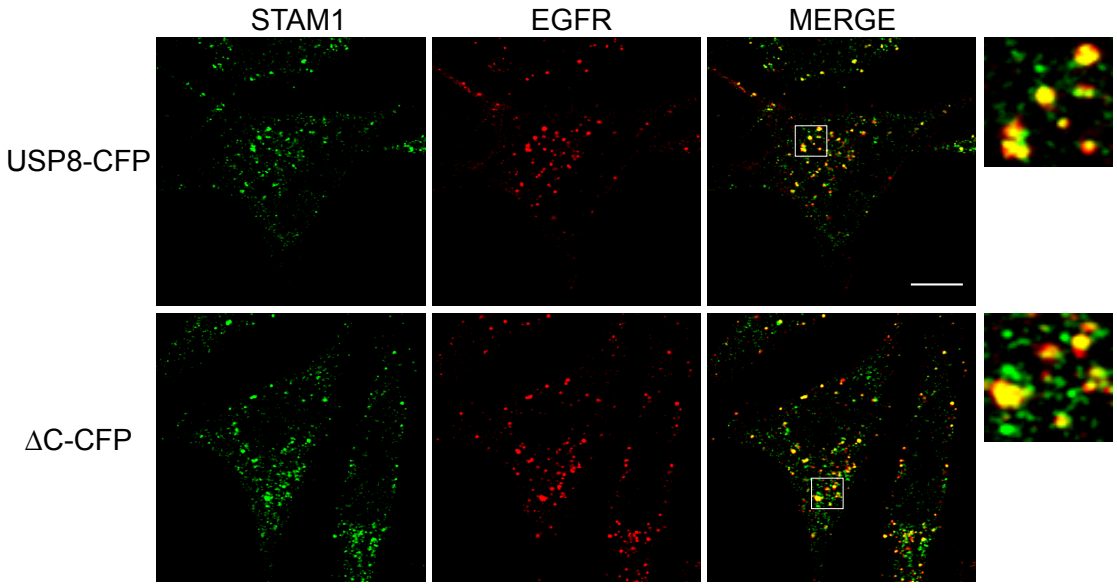
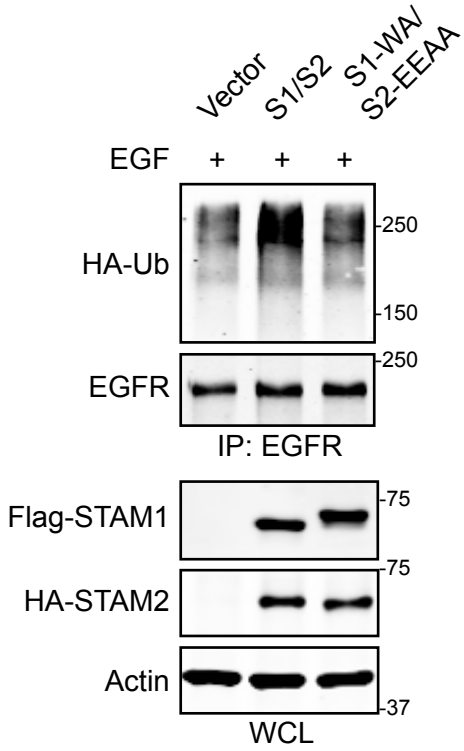

pS-Control
pS-USP8
siC
siHrs
siC
siHrs
EGF - + - + - + - +
EGFR
USP8
Hrs
Actin
250
150
100
75
37

FIG. S2

A.


STAM1
EGFR
MERGE
USP8-CFP
ΔC-CFP

B.


Vector
S1/S2
S1-WA/
S2-EEAA
EGF + + +
HA-Ub
250
150
EGFR
250
IP: EGFR
Flag-STAM1
75
HA-STAM2
75
Actin
37
WCL

C.

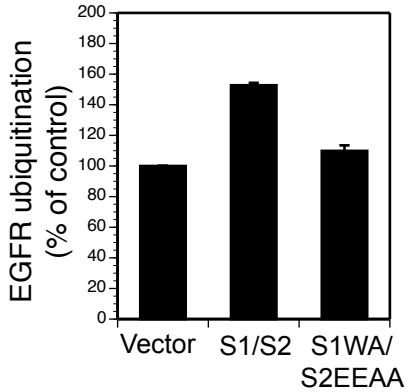
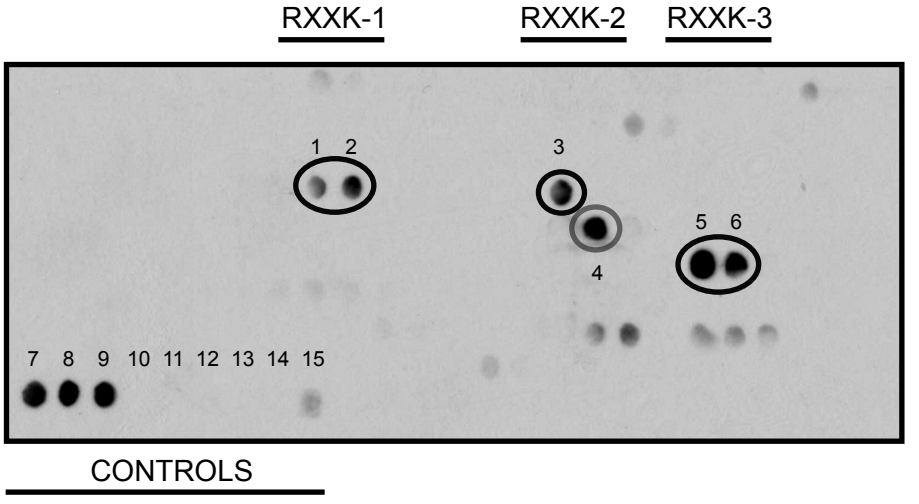

EGFR ubiquitination
(% of control)
200
180
160
140
120
100
80
60
40
20
0
Vector
S1/S2
S1WA/
S2EEAA

FIG. S3

A.



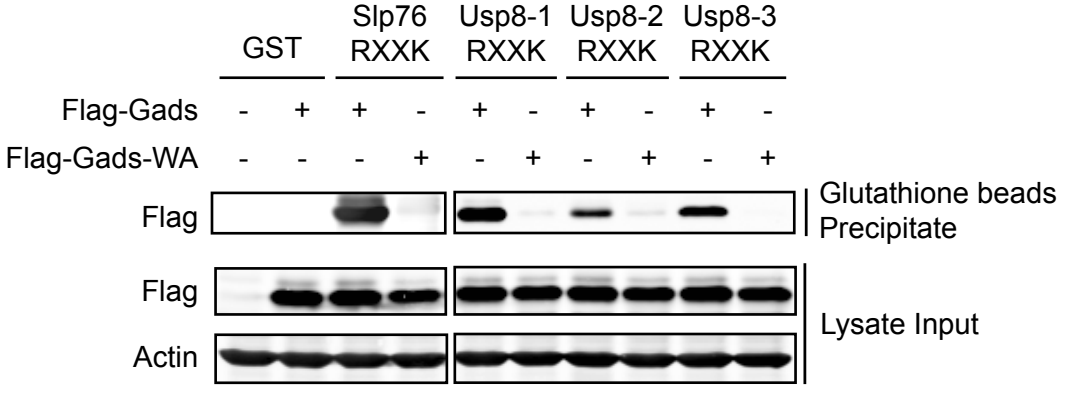
USP8 scan peptides:

1. TKSVPQVD**R**TK**K**
2. QVD**R**TK**K**PSVKL
3. SD**R**ST**K**PVFPSP
4. PVEGKR**C**PTSEA
5. PAVTPMVN**R**EN**K**
6. MVN**R**EN**K**PPCYP

USP8 control peptides:

7. PQVD**R**TK**K**PSVK
8. VLSD**R**ST**K**PVFP
9. PMVN**R**EN**K**PPCY
10. PQVD**K**TK**K**PSVK
11. VLSD**K**ST**K**PVFP
12. PMVN**K**EN**K**PPCY
13. PQVD**A**TK**K**PSVK
14. VLSD**A**ST**K**PVFP
15. PMVN**A**EN**K**PPCY

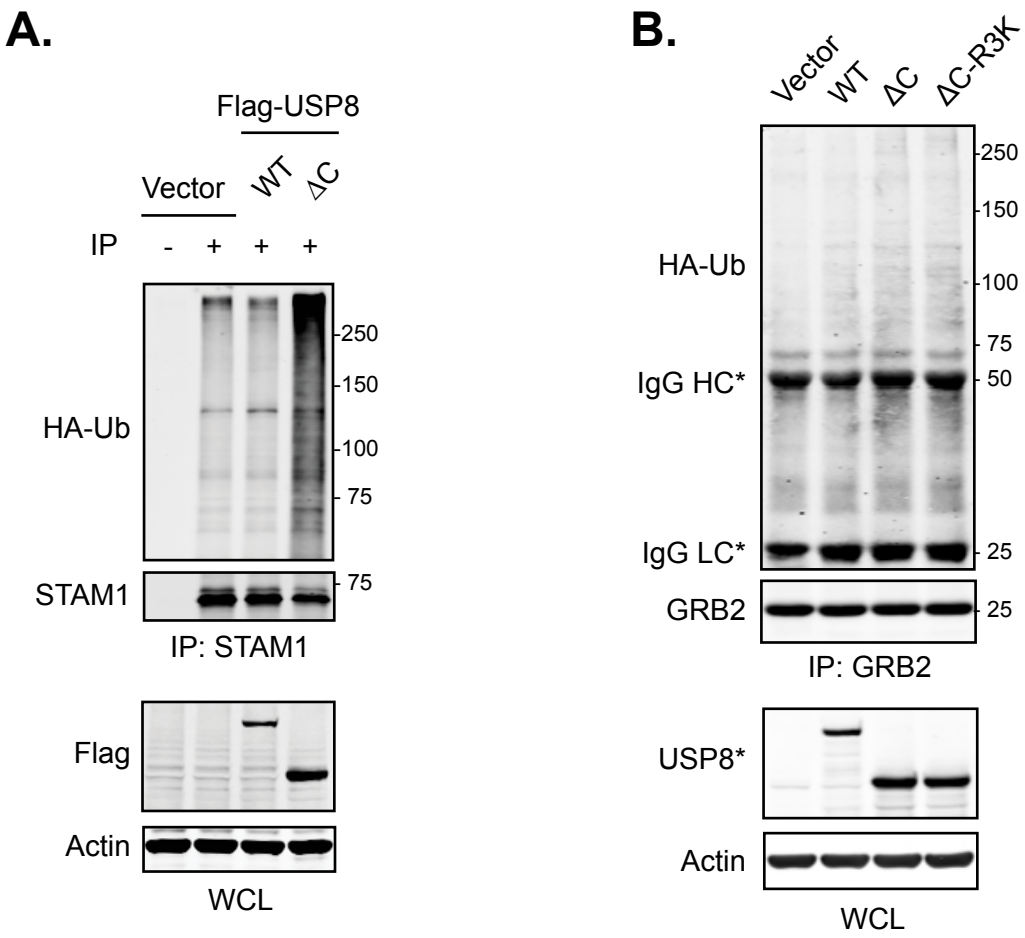
B.



C.

Domain	SLP-76 K_D (μM)	USP8-1 K_D (μM)	USP8-2 K_D (μM)	USP8-3 K_D (μM)
Gads-SH3C	0.24 ± 0.2	1.3 ± 0.1	10.2 ± 0.4	3.7 ± 0.6
Stam2-SH3	3.7 ± 0.2	10.3 ± 0.2	57 ± 1	10.7 ± 0.2
Stam1-SH3	10 ± 1	14.1 ± 0.4	74 ± 1	22.7 ± 0.4
Grb2-SH3C	12.2 ± 0.3	52 ± 1	> 280 ± 0.4	39.9 ± 0.1

FIG. S4


A.
Flag-USP8
Vector
WT
ΔC
IP
-
+
+
+
HA-Ub
250
150
100
75
STAM1
75
IP: STAM1
Flag
Actin
WCL
B.
Vector
WT
ΔC
ΔC-R3K
HA-Ub
IgG HC*
IgG LC*
250
150
100
75
50
25
GRB2
25
IP: GRB2
USP8*
Actin
WCL